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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12N 9/50, 15/57, C11D 3/386</p>	<p>A1</p>	<p>(11) International Publication Number: WO 91/16423 (43) International Publication Date: 31 October 1991 (31.10.91)</p>
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<p>(54) Title: OXIDATION STABLE DETERGENT ENZYMES</p> <p>(57) Abstract</p> <p>Novel chemically modified detergent enzymes are provided, wherein one or more methionins have been mutated into cysteins, said cysteins subsequently being chemically modified in order to confer the enzyme improved stability towards oxidative agents. A novel process for stabilizing detergent enzymes against oxidation is also provided. Furthermore, there are provided detergent compositions comprising these novel oxidation stable detergent enzymes.</p>		

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OXIDATION STABLE DETERGENT ENZYMES

TECHNICAL FIELD

This invention is within the field of oxidation stable detergent enzymes. More specifically, the present invention relates to novel chemically modified detergent enzymes, wherein one or more methionins have been mutated into cysteins, said cysteins subsequently being chemically modified in order to confer the enzyme improved stability towards oxidative agents. The present invention is also directed towards a novel process for stabilizing detergent enzymes against oxidation. Further, the present invention is directed towards a detergent composition comprising these novel oxidation stable detergent enzymes.

BACKGROUND ART

The problems related to a generally low oxidation stability are a well known major obstacle in respect to the activity of detergent enzymes. Due to the presence of bleach active ingredients, the detergent enzymes have to perform their enzymatic action in an oxidative environment, with a consequent loss of activity.

Various solutions have been proposed to the problem, but hitherto oxidation stable detergent enzymes have not been available.

The currently used detergent enzymes have been found by isolating the enzymes from nature and testing them in detergent formulations. Some detergent enzymes have been artificially modified by deletions or substitutions of amino acids within their molecule, in order to achieve novel detergent enzymes with altered chemical and enzymatic properties. Techniques as random and site-directed mutagenesis have been applied from knowledge of the physical and chemical

properties of the enzymes, and accordingly these techniques have mostly been applied to proteases.

Especially site-directed mutagenesis of the subtilisin genes has attracted much attention, and various mutations are described in patent publications (see e.g. EP 130,756; EP 214,435; EP 303,761; EP 260,105; WO 87/04461; WO87/05050).

A subtilisin is a serine protease produced by Gram-positive bacteria or fungi. A wide variety of subtilisins have been identified, and in several cases the amino acid sequence has been determined (WO 89/06279 and DK Patent Application No. 3169/89). In position 222 these wildtype subtilisins hold a methionyl residue, and this methionyl residue is identified as being responsible for the lability of the enzyme towards oxidative agents (J. Biol. Chem., 244, 5333-5338 (1969)).

Substitution of the residue at position 222 with any other of the 19 essential amino acid residues has been carried out, and the mutants obtained were investigated in respect to relative activity (J. Biol. Chem., 260, 6518-6521 (1985)). Only the Cys-mutant displayed a relative activity in magnitude of the wildtype (56%), but similar to the wildtype this Cys-mutant was instable to oxidative agents, and a "half life" in the order of 12 minutes in 1 M H_2O_2 was found (the wildtype-subtilisins do not contain cystein residues). Among the most oxidation stable mutants the Ala-mutant displayed the highest enzymatic activity (11%), and it did not loose its activity even after 15 minutes in 1 M H_2O_2 .

For scientific use it has been demonstrated that site-directed mutagenesis can be combined with chemical modifications in order to achieve enzyme derivatives with altered properties. Thus, a chemical modification of a cysteinyl residue introduced in the binding site of carboxypeptidase-Y has proved to give enzyme derivatives that are more effective in deamidations of peptide amides and peptide synthesis, respectively (Bech, L.M. & Breddam, K.; Carlsberg Res. Commun., 53, 389-393 (1988)).

Therefore, it is an object of the present invention to provide novel chemically altered detergent enzymes, exhibiting improved oxidation resistance, and at the same time substantially retaining their proteolytic activity in respect of their wash performance. Further, it is an object of the present invention to establish a novel process for stabilizing detergent enzymes against oxidation.

BRIEF DISCLOSURE OF THE INVENTION

By way of chemical modification of variants of detergent enzymes, the preparation of novel active chemically modified detergent enzymes, with conferred stability towards oxidative agents, has now surprisingly succeeded.

Thus, according to a first aspect, the present invention provides novel oxidation stable chemically modified detergent enzymes, wherein one or more methionins have been mutated into cysteins, and the cysteins are subsequently modified chemically in order to substitute the hydrogen of the HS-group into a group of the general formula R^1S- , wherein R^1 is C_{1-6} -alkyl.

According to a second aspect, the present invention provides a novel process for stabilizing detergent enzymes against oxidation, whereby a variant of the detergent enzyme, wherein one or more methionins have been mutated into cysteins, are chemically modified by substitution of the hydrogen of the HS-group of the cysteins into a group of the general formula R^1S- , wherein R^1 is C_{1-6} -alkyl, due to reaction with a compound of the general formula $R^1SSO_2R^2$, wherein R^1 is C_{1-6} -alkyl and R^2 is C_{1-4} -alkyl, and the reaction is carried out at pH values in the range 5 to 11.

According to a third aspect, the present invention provides a detergent composition comprising one or more of the oxidation stable detergent enzymes, provided in the form of a detergent additive, preferably a non-dusting granulate or a stabilized liquid.

BRIEF DESCRIPTION OF DRAWINGS

The invention will be explained in more detail in the following with reference to the figures, wherein

Fig. 1 shows a schematic representation of the chemical modification reaction of the invention in general,

Figs. 2a-d show a comparison of the structures of various groups attached to the peptide backbone of the detergent enzyme.

Fig. 2a shows a "wild-type" enzyme with a methionine sidechain.

Fig. 2b shows an enzyme variant, wherein a methionine has been mutated into a cysteine (M→C).

Fig. 2c shows chemically modified enzyme variants of the invention.

Fig. 2d shows an oxidated "wild-type" enzyme.

Figs. 3-5 show the relation between wash performance and enzyme concentration in three different detergent compositions for the wild type enzyme (S. 309), the methionine substituted enzyme (M222C), and a chemically modified enzyme according to this invention (m-M222C).

DETAILED DISCLOSURE OF THE INVENTION

By a chemical modification process according to the present invention, detergent enzymes, wherein one or more methionins have been mutated into cysteins, are treated with an agent of the general formula $R^1SSO_2R^2$, wherein R^1 and R^2 are defined below, in order to substitute the hydrogen of the HS-group of the cysteine into a group of the general formula R^1S- (cf. Fig. 1). By this process, cysteine is changed into an amino acid that sterically resembles the methionine originally present, but is much more stable towards oxidative agents than methionine (cf. Fig. 2), and an oxidation stable detergent enzyme of the invention is obtained.

Detergent enzymes

In the detergent industry, enzymes have been implemented in washing formulations for more than 20 years. Detergent enzymes comprise amylases, lipases, cellulases and proteases, as well as other enzymes, or mixtures thereof. Commercially most important detergent enzymes are proteases, including subtilisins.

Process for oxidation stabilization

By the process for stabilizing detergent enzymes against oxidation of the invention, a variant of the detergent enzyme, wherein one or more methionins have been mutated into cysteins, are chemically modified by substitution of the HS-group of the cysteins into a group of the general formula R^1SS- , wherein R^1 is C_{1-6} -alkyl, due to reaction with a compound of the general formula $R^1SSO_2R^2$, wherein R^1 is C_{1-6} -alkyl, and R^2 is C_{1-4} -alkyl.

Starting compounds

In the process for stabilizing detergent enzymes against oxidation of the invention, starting compounds are variants of detergent enzymes, wherein one or more methionins have been mutated into cysteins.

The starting compounds can be obtained using e.g. site-directed mutagenesis. Cloning procedures and in vitro mutagenesis can be carried out essentially as described in the International Publication, WO 89/06279.

Preferred starting compounds are amylases, lipases, cellulases or proteases. More preferred starting compounds are subtilisins.

A survey of the amino acid sequence of various subtilisin proteases is given in DK Patent Application No. 3169/89. None of these subtilisins carry a cysteinyl residue, whereas they do carry one or more methionyl residues. Thus, in position 222, and adjacent to the active serine, subtilisin proteases hold a methionyl residue, and as previously men-

tioned, this residue has been identified as being responsible for the lability of the enzyme towards oxidative agents. For comparison, selected partial amino acid sequences for various subtilisin proteases are listed in Table 1. For the sake of
 5 clarity, only the residues in the region around the active serine are listed.

Table 1

COMPARISON OF AMINO ACID SEQUENCE FOR VARIOUS
SUBTILISIN PROTEASES

10	No:	210	220
	a)	P-G-V-S-I-Q-S-T-L-P-G-N--K--Y-G-A-Y-N-G-T-S-M-A-S-P-H-	
	b)	P-G-V-S-I-Q-S-T-L-P-G-G--T--Y-G-A-Y-N-G-T-S-M-A-T-P-H-	
	c)	P-G-V-S-I-Q-S-T-L-P-G-G--T--Y-G-A-Y-N-G-T-S-M-A-T-P-H-	
	d)	P-G-V-S-I-Q-S-T-L-P-G-G--T--Y-G-A-Y-N-G-T-S-M-A-T-P-H-	
15	e)	P-G-V-S-V-Y-S-T-Y-P-S-N--T--Y-T-S-L-N-G-T-S-M-A-S-P-H-	
	f)	P-G-A-G-V-Y-S-T-Y-P-T-N--T--Y-A-T-L-N-G-T-S-M-A-S-P-H-	
	g)	P-G-A-G-V-Y-S-T-Y-P-T-S--T--Y-A-T-L-N-G-T-S-M-A-S-P-H-	
	h)	P-G-V-N-V-Q-S-T-Y-P-G-S--T--Y-A-S-L-N-G-T-S-M-A-T-P-H-	
	i)	P-G-V-N-V-N-S-T-Y-T-G-N--R--Y-V-S-L-S-G-T-S-M-A-T-P-H-	
20	j)	P-G-S-W-I-Y-S-T-Y-P-T-S--T--Y-A-S-L-S-G-T-S-M-A-T-P-H-	
	k)	P-G-T-S-I-L-S-T-W-I-G-G--S--T-R-S-I-S-G-T-S-M-A-T-P-H-	
	l)	P-G-A-S-I-P-S-A-W-Y-T-S-D-T-A-T-Q-T-L-N-G-T-S-M-A-T-P-H-	
	m)	P-G-V-N-V-Q-S-T-Y-P-G-S--T--Y-A-S-L-N-G-T-S-M-A-T-P-H-	
	n)	P-G-T-D-I-K-S-T-W-N-D-G-R-T-K-I-I-S--*--G-T-S-M-A-S-P-H-	
25	o)	P-G-T-D-I-L-S-T-W-I-G-G-S-T-R-S-I-S--*--G-T-S-M-A-T-P-H-	

Table 1, continued..

	a =	subtilisin BPN' (Wells et al, 1983, <u>supra</u>)
	b =	subtilisin amylosacchariticus (Kurihara et al, 1972, <u>supra</u>)
5	c =	subtilisin 168 (Stahl and Ferrari, 1984, <u>supra</u>)
	d =	subtilisin mesentericopeptidase (Svendsen et al, 1986, <u>supra</u>)
	e =	subtilisin DY (Nedkov et al, 1985, <u>supra</u>)
	f =	subtilisin Carlsberg (Smith et al, 1968, <u>supra</u>)
10	g =	subtilisin Carlsberg (Jacobs et al, 1985, <u>supra</u>)
	h =	subtilisin 309 (International Patent Application No. PCT/DK 88/00002)
	i =	subtilisin 147 (International Patent Application No. PCT/DK 88/00002)
15	j =	thermitase (Meloun et al, 1985, <u>supra</u>)
	k =	proteinase K (Betz et al, 1988, Eur. J. Biochem. 178: 155 ff), and Gunkel et al, 1989, Eur. J. Biochem. 179: 185 ff)
	l =	aqualysin (Kwon et al, 1988, Eur. J. Biochem. 173:491 ff)
20	m =	Bacillus PB92 protease (European Patent Publication No. 0 283 075)
	n =	Protease TW7 (Tritirachium album)(Internataional Patent Application No. PCT/US88/01040)
25	o =	Protease TW3 (Tritirachium album)(Internataional Patent Application No. PCT/US88/01040)

* = assigned deletion

Among the subtilisin variants, wherein M222 has been substituted with cystein, those preferred are subtilisin-309-M222C, subtilisin-147-M222C, subtilisin-BPN'-M222C, subtilisin Carlsberg-M222C and proteinase K-M222C. Most preferred is subtilisin-309-M222C.

Chemical modification

Modifying chemical reagents of the general formula $R^1SSO_2R^2$ are commercially available, or can be prepared by traditional organic chemical methods (see "materials and methods").

For sterical reasons, the substituents R^1 and R^2 have to be of limited size. Therefore, preferred R^1 -substituents are C_{1-6} -alkyl, and most preferred are methyl, ethyl or propyl/isopropyl. Preferred R^2 -substituents are C_{1-4} -alkyl, and most preferred are methyl or ethyl.

Preferred reagents of the general formula $R^1SSO_2R^2$ are methane-methyl-thiosulphonate (MTS), ethane-methyl-thiosulphonate (EMTS) and propane-methyl-thiosulphonate (PMTS) (see "materials and methods").

The pH should be in the range 5 to 11, and most preferred in the range 8 to 10.

Products

The novel oxidation stable chemically modified detergent enzymes of the invention are detergent enzymes, wherein one or more methionins have been mutated into cysteins, followed by chemically modification of the cysteins, in order to substitute the hydrogen of the HS-group into a group of the general formula R^1S- , wherein R^1 is C_{1-6} -alkyl.

Preferred oxidation stable detergent enzymes of the invention are amylases, lipases, cellulases or proteases, wherein one or more methionins have been mutated into cysteins in order to substitute the hydrogen of the HS-group into a group of the general formula R^1S- , wherein R^1 is C_{1-6} -alkyl.

More preferred oxidation stable detergent enzymes of the invention are subtilisins.

Most preferred oxidation stable detergent enzymes of the invention are amylases, lipases, cellulases or proteases, wherein one or more methionins have been mutated into cysteins in order to substitute the hydrogen of the HS-group into a group of the general formula R^1S- , wherein R^1 is C_{1-3} -alkyl.

In another embodiment of the invention, preferred oxidation stable detergent enzymes are chemically modified subtilisins, wherein the methionine in position 222, adjacent to the active serine, has been mutated into cysteine, followed
5 by chemical modification, in order to substitute the hydrogen of the HS-group of the cysteins into a group of the general formula R^1S- , wherein R^1 is C_{1-6} -alkyl.

More preferred are oxidation stable chemically modified subtilisins, where the subtilisins are subtilisin-
10 309, subtilisin-147, subtilisin BPN', subtilisin Carlsberg or proteinase K.

Most preferred is a oxidation stable chemically modified subtilisin, which is subtilisin-309.

In the context of this application, a specific
15 notation for the chemically modified detergent enzymes of the invention is used. According to that notation, the M222C variant of subtilisin-309 (subtilisin-309-M222C), which subsequently has been chemically modified through reaction with $R^1SSO_2R^2$, is designated subtilisin-309-M222C-SR¹. If the
20 reagent is MMTS, the notation will be subtilisin-309-M222C-SM. If the reagent is EMTS, the notation will be subtilisin-309-M222C-SE. If the reagent is PMTS, the notation will be subtilisin-309-M222C-SP, and so forth.

According to the invention, any detergent enzyme can
25 be modified chemically in order to achieve an improved oxidation stability. Preferred oxidation stable chemically modified detergent enzymes according to the invention are subtilisin-309-M222C-SM and subtilisin-309-M222C-SP.

Comparative results from the example are shown in the
30 following Table 2.

Table 2
Properties of subtilisin-309 variants

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}mM^{-1}$)	rel. activity*	$t_{1/2}$ (min.)	rel. $t_{1/2}$ (min.)
Subtilisin-309 (wild type)	1.76±0.06	91.2±1.0	52	100%	60	100%
Subtilisin-309 in oxidized state	2.36±0.09	6.10±0.07	2.6	5%	-	-
Subtilisin-309-M222C	1.82±0.14	43.2±0.03	23.8	46%	15	39%
Subtilisin-309-M222C-SM	2.02±0.08	59.0±0.8	29	56%	>500	89%
Subtilisin-309-M222C-SP	2.22±0.12	15.7±0.4	7.1	14%	>500	-

*Substrate: Suc-Ala-Ala-Pro-Phe-pNA

**Substrate: DMC (Dimethyl-casein)

- = not measured

As appears from the table, K_m is nearly constant for all enzymes, whereas k_{cat} varies considerably. The "wild type" enzyme possesses the highest specific activity, but evidently, the subtilisin-309-M222C-SM enzyme possesses the best stability towards oxidative environments along with relatively high enzymatic activity.

The non-modified subtilisin-309-M222C variant possesses poor enzymatic activity as well as poor stability.

Detergent compositions

The detergent composition of the invention may comprise one or more surfactants which may be of an anionic, non-ionic, cat-ionic, amphoteric or zwitter-ionic type, or a mixture of these. Typical examples of anionic surfactants are linear alkyl benzene sulfonates (LAS); alkyl sulfates (AS); alpha olefin sulfonates (AOS); alcohol ethoxy sulfates (AES) and alkali metal salts of natural fatty acids. Examples of non-ionic surfactants are alkyl polyethylene glycol ethers; nonylphenol polyethylene glycol ethers; fatty acids esters of sucrose and glucose; and esters of polyethoxylated alkyl glucoside.

The detergent composition of the invention may also contain other detergent ingredients known in the art such as builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, stabilizers for the enzymes and bleaching agents, formulations aids, optical brighteners, foam boosters, chelating agents, fillers, fabric softeners, etc. The detergent composition of the invention may be formulated substantially as described in J. Falbe [Falbe, J.; Surfactants in Consumer Products. Theory, Technology and Application; Springer Verlag 1987, vide in particular the section entitled "Frame formulations for liquid/powder heavy-duty detergents"].

It is at present contemplated that the detergent composition of the invention may contain the enzyme prepara-

tion in an amount corresponding to 0.0005-0.5 CPU of the proteolytic enzyme per litre of washing liquor.

The detergent compositions of the invention can be formulated in any convenient form such as powders, liquids, etc.

The detergent composition of the invention may advantageously include one or more other enzymes, e.g. lipases; amylases; cellulases; and/or peroxidases, conventionally included in detergent compositions.

The protease of the invention may be included in a detergent composition by adding separate additives containing the detergent protease, or by adding a combined additive comprising different detergent enzymes.

The additive of the invention can be formulated e.g. as granulates, liquids, slurries, etc. Preferred detergent additive formulations are non-dusting granulates or stabilized liquids. Dust free granulates may be produced e.g. according to GB 1,362,365 or US 4,106,991, and may optionally be coated by methods known in the art. The detergent enzymes may be mixed before or after granulation. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as e.g. propylene glycol; a sugar or sugar alcohol; lactic acid or boric acid, according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

In useful embodiments the protease of the invention may be incorporated in detergent formulations according to e.g. EP 342,177; EP 368,575; EP 378,261; and EP 378,262.

From studies in wash performance it is demonstrated that chemically modified subtilisin variants according to the present invention possess washability in magnitude at least of that of the "wild-type" subtilisin and the non-modified M222C-variant.

The wash performance is tested in examples 3 to 5 ?, and the results are set up in Figs. 3-5.

Due to a novel chemical modification process according to the present invention it is therefore possible to obtain novel subtilisin variants. These novel chemically modified subtilisin variants according to the present invention possess excellent stability towards oxidative agents as well as a relatively high enzymatic activity. Moreover, their wash performance is retained in comparison to their parent enzymes.

Materials and methods

Modifying chemical reagents of the general formula $R^1SSO_2R^2$ are commercially available, or can be prepared by traditional organic chemical methods from a common precursor, sodium methanethiolsulphonate, obtained by reaction of methane sulfonyl chloride and sodium sulphide, as described by Shaked et al.; Biochemistry; 19, 4156-4166 (1980). MMTS, EMTS and PMTS e.g. are then synthesized by reaction of sodium methanethiolsulphonate with bromomethane, bromoethane and bromopropane, respectively.

The extent of the modifying chemical reaction can be monitored by e.g. Ellman's reaction, applied on the modified subtilisin variant. This reaction is carried out as described in the example.

The proteolytic activity can be monitored spectrophotometrically at 25°C, following the degradation of the peptide substrates Suc-Ala-Ala-Pro-Phe-pNA (Suc = Succinyl and pNA = p-nitro-anilid) or DMC (dimethyl-casein), and subsequent calculation of the kinetic parameters k_{cat} and K_M .

The proteolytic activity can also be determined by the dimethyl-casein (DMC) method, described in publication AF 101/4-gb (or later editions), available on request to NOVO NORDISK A/S, Denmark, which publication is hereby included by reference.

$t_{1/2}$ is the half life of the enzyme, and is measured in 0.1 M H_2O_2 , pH 6.5 at approximately 25°C by the decrease of proteolytic activity.

The following examples will further illustrate the present invention. The examples demonstrating certain specific embodiments of the invention should not be interpreted as limiting for the scope of this invention which should be defined by the appended claims in conjunction with this specification and examples.

EXAMPLE 1

Chemical modification of subtilisin-309-M222C-variant

Subtilisin-309-M222C variant was produced as indicated in International Patent Publication No. WO 89/06279, which is hereby incorporated by reference, and kept in a buffer solution consisting of 10 mM MES (2-[N-morpholino]ethanesulphonic acid), 200 mM boric acid, 2 mM CaCl_2 , < 0.1 M NaCl, with pH 6.5. The enzyme was desalted on a P4 gel filtrating column (from Bio Rad), equilibrated with 5 mM MES and 2 mM CaCl_2 at pH 6.5.

The modification was carried out by adding to 6.2 ml M222C, $\text{Abs}_{280} = 9.7$, 0.55 ml 1 M CHES (2-[N-cyclohexylamino]ethanesulphonic acid), pH 9.5 (from SIGMA) and 0.25 ml 943 mM methane-methyl-thiosulphonate (MMTS) (from Aldrich-Chemie) in ethanol. This mixture was left at room temperature for 60 min. The mixture was then desalted on a 48 ml P4 column, equilibrated with 5 mM MES and 2 mM CaCl_2 , with pH 6.5. Fractions from the column with highest absorbance were pooled, 10 ml, $\text{Abs}_{280} = 5.79$, 95% yield.

To ensure that the cystein had reacted, Ellman's reaction was carried out on the modified enzyme. During this reaction, Ellman's reagent, 2,2-dinitro-5,5-dithiodibenzoic acid, reacts with the free SH-group, and a nitro-mercaptobenzoic acid is released. This liberation can be monitored at 412 nm, where $\Delta\epsilon = 13600 \text{ M}^{-1}\text{cm}^{-1}$.

The enzyme was denatured by making the solution 0.1 M in respect to HCl, followed by freeze drying and dissolution

in a buffer solution, consisting of 50 mM Bicine (N,N-bis[2-hydroxyethyl]-glycine), 2 mM CaCl_2 and 0.1 M KCl, with pH 8.5. 900 μl of this solution were pipetted into a cuvette, and 900 μl of the buffer solution were used as a reference. Abs_{280} was used to measure the concentration of enzyme. 10 μl of 10 mM Ellman's reagent in methanol were added to both cuvettes. The change in absorbance at 412 nm relates to the number of free SH-groups. In the reaction product, subtilisin-309-M222C-SM, no free SH-groups exist, meaning that the introduced cystein in subtilisin-309-M222C had become modified. In subtilisin-309-M222C, used as a reference, the introduced cystein was recovered due to reaction of the free SH-group with Ellman's reagent (90%). This demonstrates that cystein had become modified in the reaction product subtilisin-309-M222C-SM.

As a further control, the enzyme was titrated with phenyl-Hg-Cl. When subtilisin-309-M222C is modified with phenyl-Hg-Cl, the activity decreases. When the ratio phenyl-Hg-Cl/WT exceeds 1, the activity constantly remains 10% of the activity of the non-modified enzyme, corresponding to 1 group per enzyme being modified. In a similar reaction of the product subtilisin-309-M222C-SM with phenyl-Hg-Cl, no activity change is observed, even in the case of an excess of phenyl-Hg-Cl relative to subtilisin-309-M222C-SM of 2.5. This demonstrates that the free cystein in subtilisin-309-M222C has become modified in the product subtilisin-M222C-SM.

EXAMPLE 2

Comparative characterisation

For kinetic characterization, the savinase enzymes were assayed in:

50 μl enzymatic solution
900 μl 50 mM Bicine, 2 mM CaCl_2 , 0.1 M KCl, pH 8.5
50 μl X mM Suc-Ala-Ala-Pro-Phe-pNA in DMF

wherein the cleavage of substrate between Phe and pNA (p-nitro-anilid) is monitored spectrophotometrically at 410 nm, $\Delta\epsilon=8500\text{M}^{-1}\text{cm}^{-1}$. The enzymatic concentrations are determined from the absorption at 280 nm, using $\Delta\epsilon_{280}$ determined from the amino acid composition, $\Delta\epsilon_{280}=23217\text{M}^{-1}\text{cm}^{-1}$.

The kinetic parameters k_{cat} and K_{M} are determined from a direct fit of the initial velocities at 7 concentrations of substrate to the Michaelis Menten equation.

The enzyme concentration, X , is varied from 4 to 131 mM, so that the concentration of the substrate in assay varies from 0.2 to 6.5 mM. Thus selected around the K_{M} value of the enzymes.

The lapse of time for the effect of 0.1 M H_2O_2 on the activity of the wild type and mutant enzymes is investigated by incubating the enzymes, approx. 0.5 μM , in 5 mM MES, 2 mM CaCl_2 , pH 6.5, with H_2O_2 (from a 30% stock solution, MERCK). At regular interval times the reaction was stopped by diluting an appropriate amount of enzyme in assay with 0.35 mM Suc-Ala-Ala-Pro-Phe-pNA, 50 mM Bicine, 2 mM CaCl_2 , 0.1 M KCl, pH 8.5, and monitoring the change in absorbance at 410 nm. The remaining activity is determined as the percentage of activity of non-treated enzyme control. The half-life, t_{h} , corresponding to 50% of activity remaining, is determined from the activity curves.

The characterisation results are set up in Table 2 shown previously.

EXAMPLE 3

Wash performance

The wash performance tests were accomplished on grass juice soiled cotton cloths and performed at 20°C, isothermically for 10 minutes.

The modified subtilisin-309-M222C-SM (m-M222C) variant was compared to the wild type enzyme (S. 309) and the non-modified subtilisin-309-M222C variant (M222C). The detergents did not contain any enzymes prior to the addition of the proteases of the invention.

In Test A 5.0 g/l of a commercial European type powder detergent (A) with perborate and activator were used. The detergent was dissolved in approx. 9°dH (German Hardness) water, and pH was measured to 10.2. The enzymes were dosed from 0.008 to 0.4 mg protein/l, vide Fig. 3.

In Test B 5.0 g/l of a commercial European type powder detergent (B) with perborate and activator were used. The detergent was dissolved in approx. 9°dH water, and pH was measured to 9.5. The enzymes were dosed from 0.008 to 0.08 mg protein/l, vide Fig. 4.

In Test C 1.1 g/l of a commercial US type powder detergent (C) with perborate and activator were used. The detergent was dissolved in approx. 6°dH water, and pH was measured to 9.5. The enzymes were dosed from 0.008 to 0.08 mg protein/l, vide Fig. 5.

Subsequent to washing, the cloths were flushed in running tap-water for 25 minutes in a bucket. The cloths were then air-dried overnight (protected against daylight), and the remission (%R) at 460 nm was determined.

As a measure of the wash performance differential remission, ΔR , was used being equal to the remission after wash with enzyme added, minus the remission after wash with no enzyme added.

From the figures it appears that the 3 enzymes perform equally well on a protein basis.

CLAIMS:

1. Oxidation stable chemically modified detergent enzymes, wherein one or more methionins have been mutated into cysteins, characterized in, that the cysteins have been
5 chemically modified in order to substitute the hydrogen of the HS-group into a group of the general formula R^1S- , wherein R^1 is C_{1-6} -alkyl.
2. Oxidation stable chemically modified detergent enzymes, according to claim 1, characterized in, that the
10 detergent enzymes are amylases, lipases, cellulases or proteases.
3. Oxidation stable chemically modified detergent enzymes according to claim 1, characterised in, that the detergent enzymes are subtilisins.
- 15 4. Oxidation stable detergent enzymes according to any of claims 1-3, characterized in, that R^1 is C_{1-3} -alkyl.
5. Oxidation stable chemically modified subtilisins, wherein the methionine in position 222, adjacent to the active serine, has been mutated into cysteine, characterized in, that
20 the cysteine has been chemically modified in order to substitute the hydrogen of the HS-group into a group of the general formula R^1S- , wherein R^1 is C_{1-6} -alkyl.
6. Oxidation stable chemically modified subtilisin according to claim 5, characterized in, that the subtilisin is
25 subtilisin-309, subtilisin-147, subtilisin BPN', subtilisin Carlsberg, or proteinase K.

7. Oxidation stable chemically modified subtilisin according to claim 5, characterized in, that the subtilisin is subtilisin-309.

5 8. Oxidation stable chemically modified subtilisin according to claims 5-7, characterized in, that R¹ is C₁₋₃-alkyl.

9. Oxidation stable chemically modified subtilisin, Subtilisin-309-M222C-SM.

10 10. Oxidation stable chemically modified subtilisin, Subtilisin-309-M222C-SP.

15 11. A process for stabilizing detergent enzymes against oxidation, characterized in, that a variant of the detergent enzyme, wherein one or more methionins have been mutated into cysteins, are chemically modified by substitution of the hydrogen of the HS-group of the cysteins into a group of the general formula R¹S-, wherein R¹ is C₁₋₆-alkyl, due to reaction with a compound of the general formula R¹SSO₂R², wherein R¹ is C₁₋₆-alkyl, and R² is C₁₋₄-alkyl, and the reaction is carried out at a pH-value in the range 5 to 11.

20 12. A process for stabilizing detergent enzymes against oxidation, according to claim 11, characterized in, that the detergent enzymes are amylases, lipases, cellulases or proteases.

25 13. A process for stabilizing subtilisins against oxidation, characterised in, that subtilisin variants, wherein one or more methionins have been mutated into cysteins, are chemically modified by substituting the hydrogen of the HS-group of the cysteins into a group of the general formula R¹S- wherein R¹ is C₁₋₆-alkyl, due to reaction with a compound of
30 the general formula R¹SSO₂R², wherein R¹ is C₁₋₆-alkyl, and R² is

C₁₋₄-alkyl, and the reaction is carried out at a pH-value in the range 5 to 11.

14. A process according to claim 13, characterized in, that the reaction is carried out at a pH-value in the
5 range 8 to 10.

15. A process according to either of claims 13-14, characterized in, that R¹ is C₁₋₆-alkyl, and R² is C₁₋₂-alkyl.

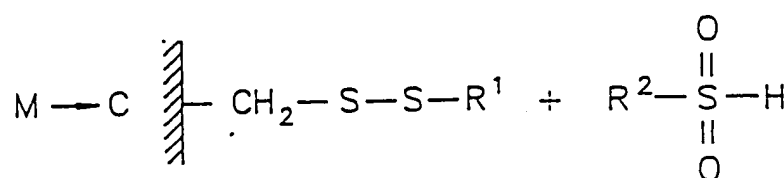
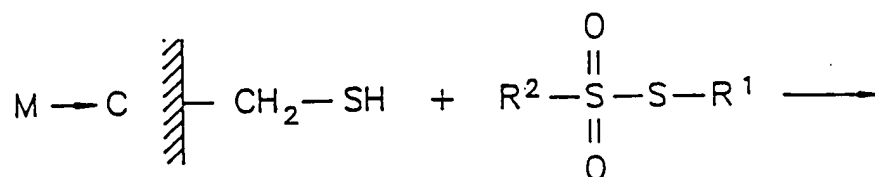
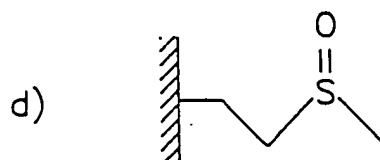
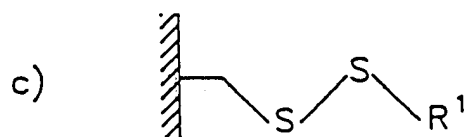
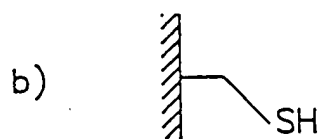
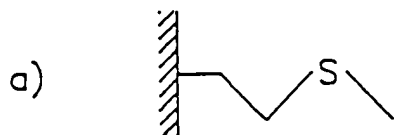
16. A process according to either of claims 13-14, characterized in, that R¹ is C₁₋₃-alkyl, and R² is C₁₋₂-alkyl.

10 17. A detergent composition comprising an oxidation stable detergent enzyme according to any of claims 1-10.

18. A detergent composition according to claim 17, which further comprises one or more other enzymes, in particular amylases; lipases; cellulases; or peroxidases.

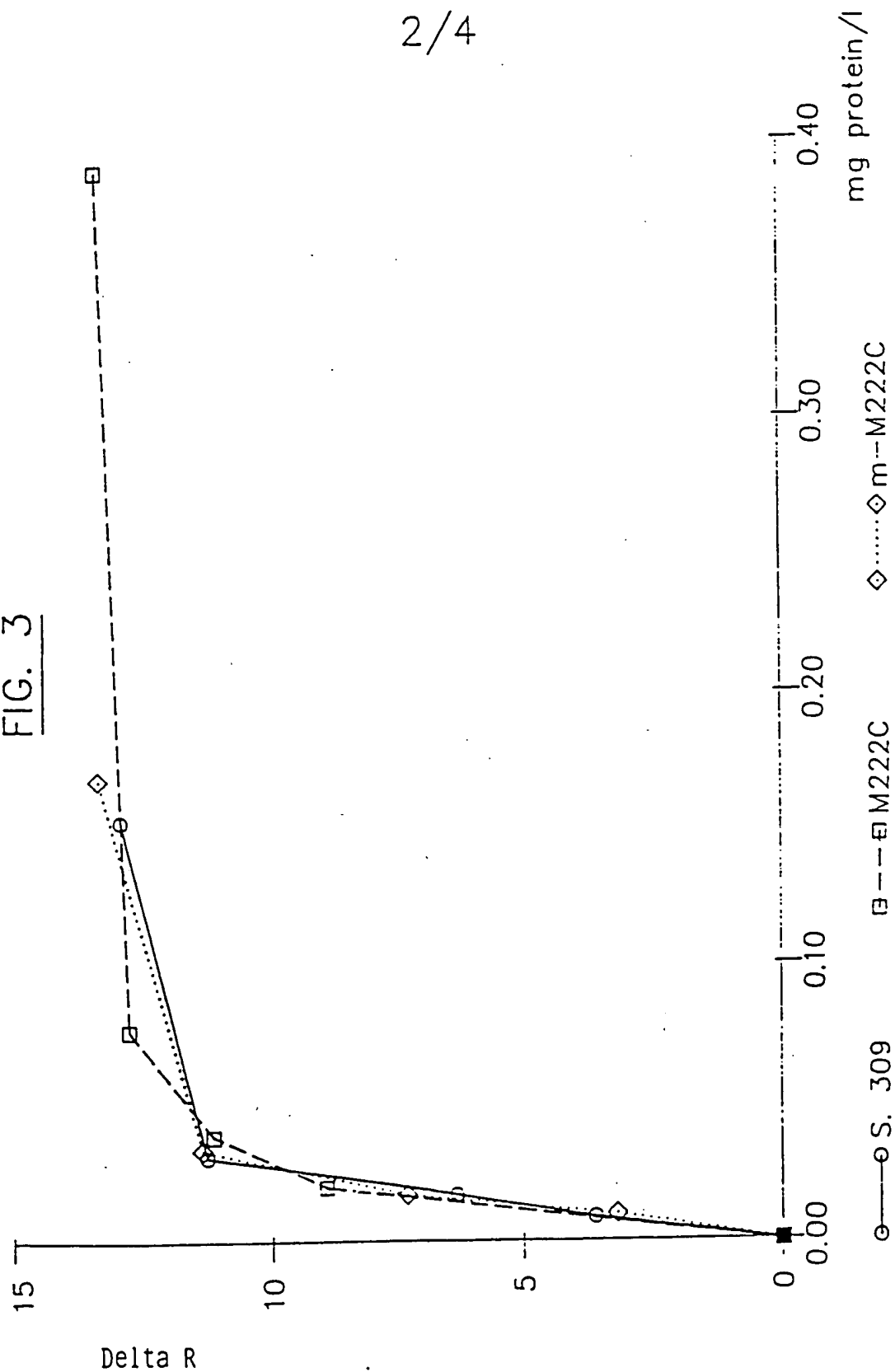
15 19. A detergent composition comprising an oxidation stable detergent enzyme according to any of claims 1-10, provided in the form of a detergent additive, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

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FIG. 1FIG. 2

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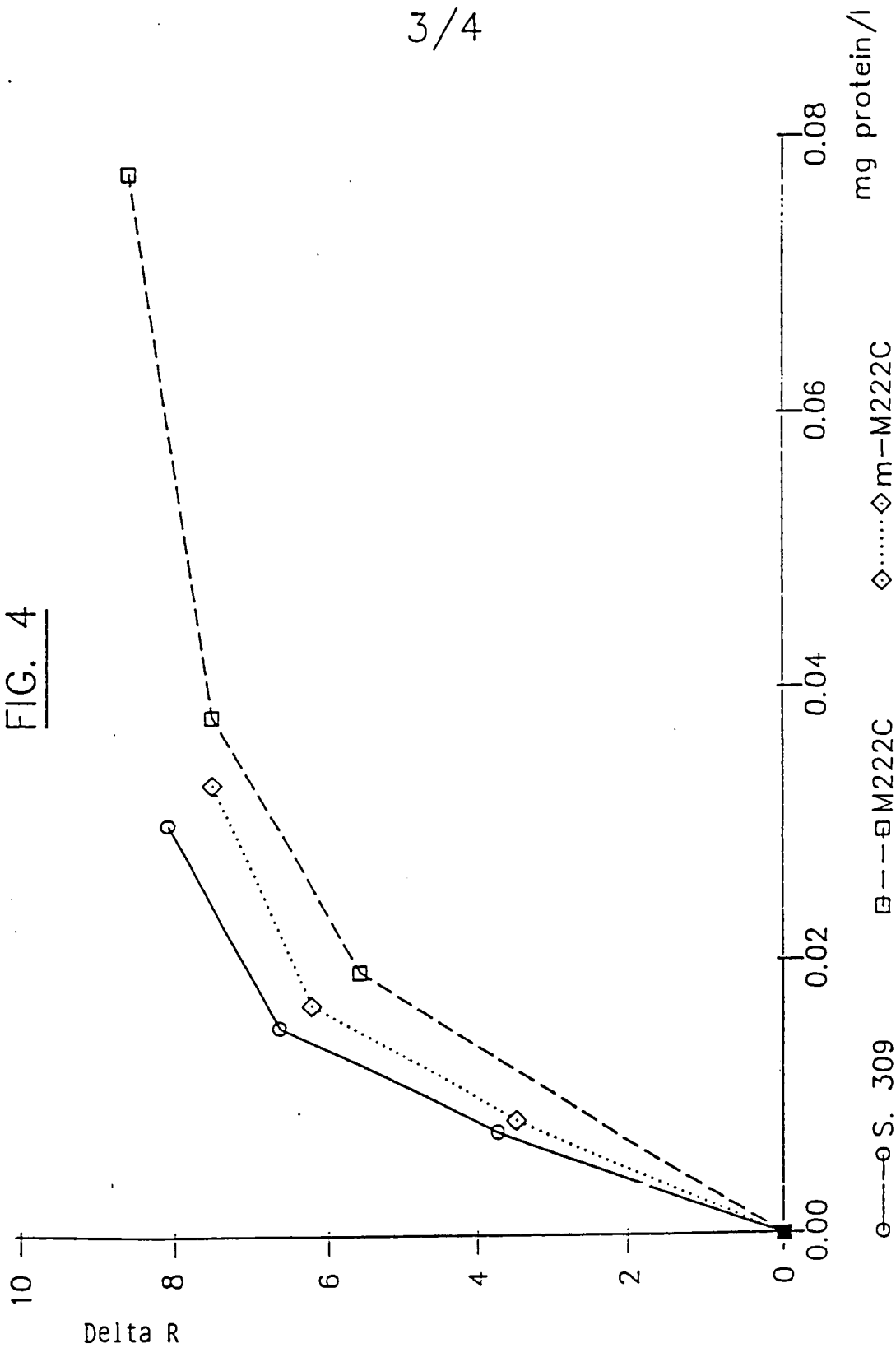
FIG. 3

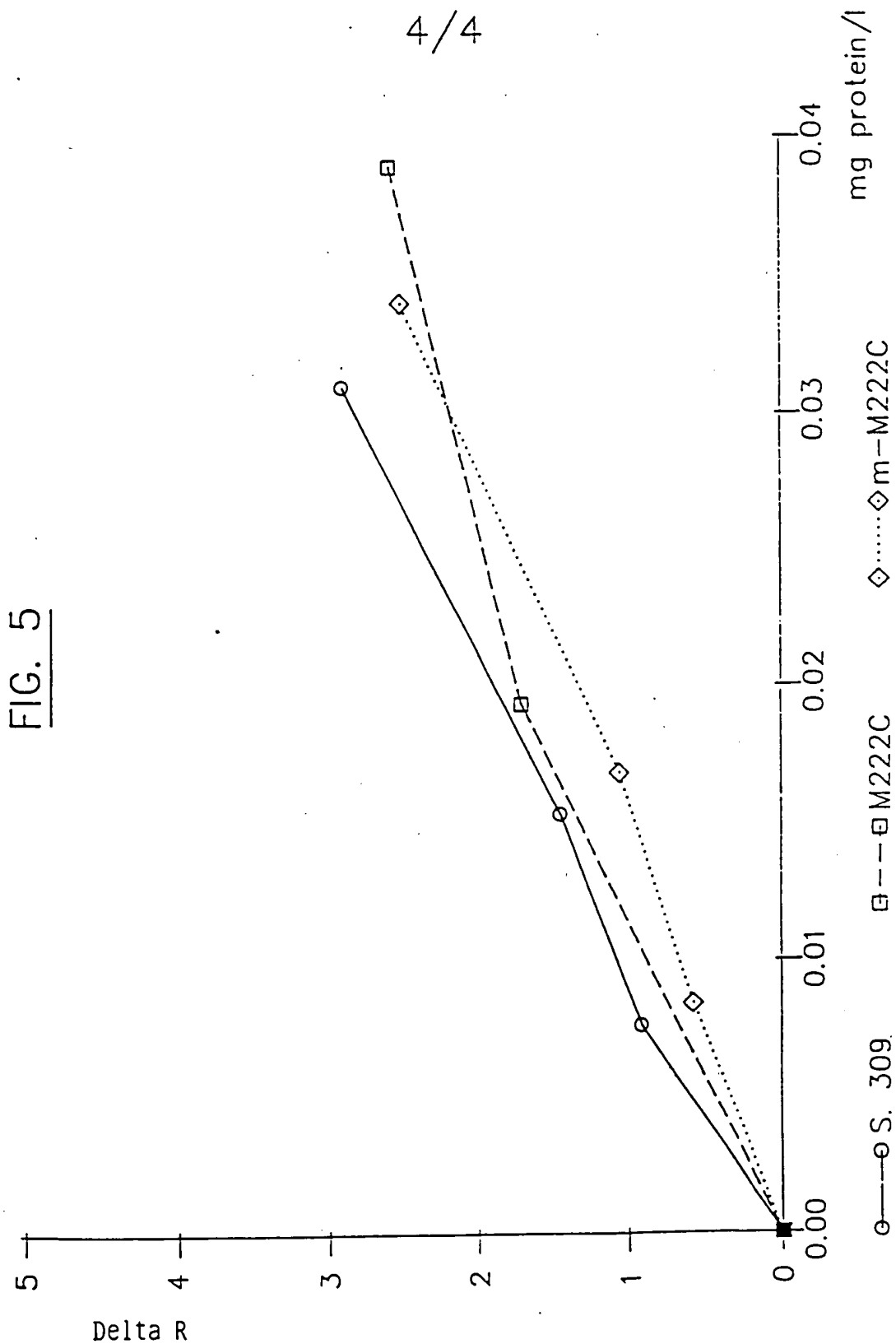


SUBSTITUTE SHEET

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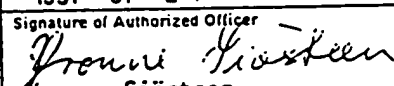
FIG. 4





INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00103

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 9/50, 15/57, C 11 D 3/386						
II. FIELDS SEARCHED <div style="text-align: right; font-size: small;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: 1px solid black; height: 40px; vertical-align: bottom;">IPC5</td> <td style="border: 1px solid black; vertical-align: bottom;">C 11 D; C 12 N</td> </tr> </table>			Classification System	Classification Symbols	IPC5	C 11 D; C 12 N
Classification System	Classification Symbols					
IPC5	C 11 D; C 12 N					
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸ SE DK,FI,NO classes as above						
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹						
Category *	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³				
P,X	Chemical Abstracts, volume 114, no. 23, 10 June 1991, (Columbus, Ohio, US), Groen et al: "A highly active and oxidation-resistant subtilisin-like enzyme produced by a combination of site-directed mutagenesis and chemical modification", see page 397, abstract 224455n, & Eur. J. Biochem. 1990, 194(3), 897-901 (Eng) <div style="text-align: center;">--</div>	1-19				
Y	The Journal of Biological Chemistry, Vol. 260, No. 11, June 1985 David A. Estell et al: "Engineering an Enzyme by Site-directed Mutagenesis to Be Resistant to Chemical Oxidation", see page 6518 - page 6521 see page 6519, right column, line 37 - line 46 <div style="text-align: center;">--</div>	1-19				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search 19th July 1991	Date of Mailing of this International Search Report 1991 -07- 24					
International Searching Authority <div style="text-align: center;">SWEDISH PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">  Yvonne Siösteen </div>					

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Carlsberg Res. Commun.. Vol. 53, 1988 Lene M. Bech et al: "CHEMICAL MODIFICATIONS OF A CYSTEINYL RESIDUE INTRODUCED IN THE BINDING SITE OF CARBOXYPEPTIDASE Y BY SITE-DIRECTED MUTAGENESIS", see page 381 - page 393 see page 386, right column 3.2. --	1-19
A	EP, A1, 0328229 (GIST-BROCADES N.V.) 16 August 1989, see abstract; claim 14 -- -----	1-19